

# The Effects of Angiopoietin-1 and -2 on Tumor Growth and Angiogenesis in Human Colon Cancer<sup>1</sup>

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## Abstract

**Angiopoietin (Ang) 1 and Ang-2 are important regulators of endothelial cell survival. Current models suggest that an increase in Ang-2 expression in endothelial cells leads to initiation of angiogenesis. We stably transfected HT29 colon cancer cells with cDNA constructs for Ang-1 or -2 or with vector alone, injected the cells s.c. into nude mice, and assessed tumor growth. Immunohistochemical analyses confirmed sustained increases of Ang-1 and -2 in the tumors. The tumors produced by the Ang-2-transfected cells were larger than the tumors produced in the other groups; those tumors also had higher vessel counts and proliferative indices than tumors in the other groups. Tumors produced by the Ang-1 transfectants had fewer vessels and lower tumor cell proliferative indices than tumors in the other groups. These data suggest that imbalances between Ang-1 and -2 that result in a net gain of Ang-2 activity lead to enhanced tumor angiogenesis and growth.**

## Introduction

Angs<sup>3</sup> are novel endothelial growth factors that are ligands for the endothelium-specific tyrosine kinase receptor Tie-2 (1). Of the four currently known Angs (Ang-1 to -4), the best characterized are Ang-1 and -2. Ang-1 binds to the Tie-2 receptor and activates it by inducing phosphorylation and dimerization of the known domains. Ang-2 also binds to Tie-2 but does not induce phosphorylation and antagonizes the action of Ang-1. VEGF and the Angs seem to play complementary and coordinated roles in the development of new blood vessels. Ang-1 helps to maintain and stabilize mature vessels by promoting interaction between endothelial cells and supporting cells (2–4). Ang-2 is expressed at sites of vascular remodeling (2) and is thought to block the stabilizing action of Ang-1. Destabilization by Ang-2 in the presence of VEGF has been hypothesized to induce an angiogenic response; however, in the absence of VEGF, Ang-2 leads to vessel regression (2, 5–7). Most of the research on Angs has focused on vasculogenesis (1, 2, 4, 8–10); few reports have focused on tumor angiogenesis (11–14), and none have investigated the role of Angs in human colon cancer. We hypothesized that an imbalance of Ang-1 and -2 in human colon cancer cells leading to a net gain of Ang-2 would lead to the initiation of angiogenesis.

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<sup>3</sup> The abbreviations used are: Ang, angiopoietin; VEGF, vascular endothelial growth factor; PCNA, proliferating cell nuclear antigen; TUNEL, terminal dUTP nick-end label; EC, endothelial cell; MTT, 1-[4,5-dimethylthiazol-2-yl]-3,5-diphenylformazan.

## Materials and Methods

**Cell Lines.** The HT29 human colon cancer cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured and maintained in MEM supplemented with 10% fetal bovine serum, 2 units/ml penicillin-streptomycin, vitamins, 1 mM sodium pyruvate, 2 mM L-glutamine, and nonessential amino acids at 37°C in 5% CO<sub>2</sub> and 95% air (15). Cells were verified to be free of *Mycoplasma*, reovirus type 3, pneumonia virus of mice, mouse adenovirus, murine hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (Microbiological Associates, Bethesda, MD).

**Subcloning of Ang-1 and -2 into pcDNA3.1 and Transfection.** The full-length cDNA for Ang-1 was a gift from Tona Gilmer, Ph.D. (Glaxo-Wellcome Inc., Research Triangle Park, NC), and the full-length cDNA for Ang-2 was a gift from Christopher D. Kontos, M.D. (Duke University, Durham, NC). These constructs were subcloned into the *Bam*HI site of pcDNA3.1 (Invitrogen, San Diego, CA), a eukaryotic expression vector driven by the human cytomegalovirus promoter containing a hygromycin resistance gene. Subcloning into the *Bam*HI restriction site yielded inserts in either the sense or antisense orientation; only inserts in the sense direction were used for the transfections. The orientation and completeness of the inserts were verified by restriction enzyme analyses and DNA sequencing (Core Sequencing Facilities, The University of Texas M. D. Anderson Cancer Center).

Vectors containing Ang-1 or -2 or the vector alone (pcDNA3.1) were transfected into HT29 cells by lipofection according to the manufacturer's protocol (Boehringer Mannheim Co., Randburg, South Africa). Selective medium containing 200 µg/ml hygromycin was added 48 h later, and viable colonies were selected and expanded. Cells from subconfluent cultures were then harvested for Northern blot analysis and *in vivo* animal experiments, as described below.

**Isolation of mRNA and Northern Blot Analysis.** Northern blot analysis was performed as described elsewhere (16). After prehybridization, the membranes were probed for Ang-1 or -2 (with full-length cDNA probes) and glyceraldehyde phosphate dehydrogenase (ATCC, Manassas, VA) as an internal control. Each cDNA probe was purified by agarose gel electrophoresis, recovered with the QIAEX gel extraction kit (Qiagen Inc., Chatsworth, CA), and radiolabeled by the random primer technique with a commercially available kit (Amersham Corp.). Nylon filters were washed at 65°C with 30 mM NaCl, 3 mM sodium citrate (pH 7.2), and 0.1% SDS. Autoradiography was then performed.

**Animals and Tumor Cell Inoculation.** Eight-week-old male nude mice were obtained from the National Cancer Institute's Animal Production Area (Frederick, MD), acclimated for 1 week while caged in groups of five. Mice were fed a diet of animal chow and water *ad libitum* throughout the experiment. Mice were randomly assigned to one of four treatment groups (10 mice per group); body weight at assignment was not different among the groups. After cell viability was verified as being ≥80% with a trypan blue exclusion test, HT29 cells (1 × 10<sup>6</sup> cells in 200 µl) were injected by means of a 30-gauge needle and a 1-ml syringe s.c. in the right flank of the animals. Tumor growth was measured every 2nd to 3rd day. Tumor volume was calculated as (diameter<sup>2</sup> × length)/2. All of the animal studies were approved by the Institutional Animal Care and Use Committee of M. D. Anderson Cancer Center. Animals in all of the four groups were killed 3 weeks after tumor cell inoculation because of the large tumors that had appeared by that time in the Ang-2-transfected group. Tumors were harvested and placed in either 10% formalin

for paraffin fixation or optimum cutting temperature solution (Miles Inc., Elkhart, IN) and snap-frozen.

**Immunohistochemical Analyses.** Antibodies for immunohistochemical analyses were obtained as follows: rat antimouse CD31/PECAM-1 antibody from PharMingen (San Diego, CA); mouse anti-PCNA clone PC10 DAKO A/S from DAKO Corp. (Carpinteria, CA); goat antihuman Ang-1 and -2 antibody from Santa Cruz Biotechnology (Santa Cruz, CA); peroxidase-conjugated goat antirat IgG (heart and lungs) and fluorescein-conjugated antigoat IgG from Jackson Research Laboratories (West Grove, PA); peroxidase-conjugated rat antimouse IgG2a from Serotec Harlan Bioproducts for Science, Inc. (Indianapolis, IN); and Alexa 594 goat antirat IgG (heart and lungs) from Molecular Probes (Eugene, OR).

Paraffin-embedded tumors were sliced in 4- to 6- $\mu$ m sections, mounted on positively charged Superfrost slides (Fisher Scientific Co., Houston, TX), and allowed to dry overnight at room temperature. Sections were deparaffinized in xylene followed by 100, 95, and 80% ethanol washes and then rehydrated in PBS (pH 7.5). These sections were used for H&E staining and detection of PCNA. Sections analyzed for PCNA were microwaved for 5 min to increase antigen retrieval.

Tumors that had been frozen in optimum cutting temperature solution were sectioned 8- to 10- $\mu$ m in thickness, mounted on positively charged slides, and air-dried for 30 min. Tissue sections were then fixed in cold acetone (5 min), followed by 1:1 acetone/chloroform (5 min) and acetone (5 min), and then washed with PBS three times, with each wash lasting 3 min. Specimens were then incubated with 3% H<sub>2</sub>O<sub>2</sub> in methanol for 12 min at room temperature to block endogenous peroxidase, washed three times (3 min each wash) with PBS (pH 7.5), and incubated for 20 min at room temperature in a protein-blocking solution consisting of PBS supplemented with 1% normal goat serum and 5% normal horse serum. The primary antibodies directed against CD31 and PCNA were diluted 1:200 and 1:50, respectively, in protein-blocking solution and applied to the sections, which were incubated overnight at 4°C. Sections were then rinsed three times (3 min each wash) in PBS and incubated for 10 min in protein-blocking solution before the addition of peroxidase-conjugated secondary antibody. The secondary antibodies used for CD31 (peroxidase-conjugated goat antirat IgG) and PCNA (peroxidase-conjugated rat antimouse IgG2a) staining were diluted 1:200 and 1:100, respectively, in protein-blocking solution. After incubating with the secondary antibody for 1 h at room temperature, the samples were washed and incubated with stable diaminobenzidine (Research Genetics, Huntsville, AL) substrate. Staining was monitored under a bright-field microscope, and the reaction was stopped by washing with distilled water. Sections were counterstained with Gill's No. 3 hematoxylin (Sigma Chemical Co., St. Louis, MO) and mounted with Universal Mount (Research Genetics) for 15 s. Treatment procedures for control specimens were similar except that the primary antibody was omitted.

**Immunofluorescent Staining for Ang, CD31, and TUNEL.** Frozen sections were stained by immunofluorescence for Ang-1 and -2 and CD31 by immunofluorescence according to the same protocol as described above, with the following modifications. After sections were incubated overnight at 4°C with the primary antibody (Ang, 1:100; Santa Cruz Biotechnology), washed, and incubated with protein-blocking solution, they were incubated for 1 h at room temperature with a secondary antibody directed against Ang-1 and -2 (fluorescein-conjugated antigoat IgG; Jackson Research Laboratories) or CD31 (Alexa 594 goat antirat IgG; Molecular Probes).

TUNEL staining was performed according to the manufacturer's protocol (Promega, Madison, WI). Briefly, the sections were fixed with 4% methanol-free paraformaldehyde; washed; permeabilized with 0.2% Triton X-100; washed; incubated with the kit's equilibration buffer; incubated with a reaction mix containing equilibration buffer, nucleotide mix, and the terminal deoxynucleotidyl transferase enzyme at 37°C for 1 h; incubated for 15 min at room temperature with 2 $\times$  SSC to stop the terminal deoxynucleotidyl transferase reaction, washed, and stained with 4,6-diamidino-2-phenylindole-2HCl (to visualize the nuclei), after which glass coverslips were applied.

**Quantification of CD31 (Tumor Vessels) and PCNA (Tumor Cell Proliferation).** The numbers of tumor vessels and PCNA-positive cells was counted by light microscopy in three random 0.159-mm<sup>2</sup> fields at  $\times$ 10 magnification with a Sony three-chip camera (Sony, Montvale, NJ) mounted on a

Zeiss universal microscope (Carl Zeiss, Thornwood, NY) and Optimas image analysis software (Bioscan, Edmond, WA) installed in a Compaq computer with a Pentium chip, a frame grabber, an optical disc storage system, and a Sony color printer. We quantified apoptosis by immunofluorescence by imaging sections digitally and processing them with Adobe Photoshop software (Adobe Systems, Mountain View, CA) as follows. CD31-positive ECs were detected by localized red fluorescence with a rhodamine filter. Tumor cell and EC apoptosis was determined by localized green fluorescence (for tumor cells) or green with red fluorescence (for ECs) with a fluorescein filter. Nuclei were detected by blue fluorescence of the 4,6-diamidino-2-phenylindole-2HCl with its respective filter. Apoptotic cells were counted in five random 0.011-mm<sup>2</sup> fields per slide.

**Cell Viability Assay.** Two thousand cells were plated in 96-well plates. At 24, 48, and 72 h the MTT assay was done as follows: 400  $\mu$ l of 2.5 mg/ml solution of MTT was added to wells and incubated for 2 h at 37°C. The supernatant was removed, and the reaction was stopped with DMSO, 100  $\mu$ l/well. The plates were placed on a shaker for 1 min, and the absorbance was determined on a plate reader at 570  $\lambda$ . Each assay was repeated four times.

**Statistical Analysis.** Body weight, tumor volume, and the number of CD31- and PCNA-positive cells were compared by using unpaired Student's *t* tests (InStat for Macintosh; GraphPad Software, San Diego, CA). Densitometric analysis was performed (Image Quant software; Molecular Dynamics, Sunnyvale, CA) to quantify the results of Northern blot analyses in the linear range of the film. Glyceraldehyde phosphate dehydrogenase mRNA was used as an internal control for loading.

## Results

**Northern Blot Analysis.** Six clones each were isolated from the Ang-1 and -2, and vector-only transfected cell cultures. An isolated clone for Ang-1 transfectants and the pooled clones for Ang-2 transfectants demonstrated the highest expression of exogenous Ang-1 and -2, respectively. By Northern blot analysis parental HT29 colon cancer cells expressed endogenous Ang-2 but not -1 (although, Ang-1 was expressed by reverse transcription-PCR). On the basis of densitometry readings, the Ang-2 pooled transfectants had eight times greater exogenous Ang-2 production than endogenous Ang-2 production. The fold increase for Ang-1 could not be calculated because of the fact that endogenous Ang-1 was not detected in the parental cell line. Ang-1 had 10 times greater exogenous Ang-1 production when compared with the other selected clones. The pooled empty vector transfectants were used for *in vivo* experiments.

**Tumor Growth and Body Weight.** Body weight was no different between the treatment and control groups before or after the treatment. Tumors in the mice treated with the Ang-2-transfected HT29 cells were significantly larger (Fig. 1A) and heavier (Fig. 1B) than those in the other groups. All of the mice were killed on treatment day 21 because the tumors in the Ang-2 group at that time were the maximum size allowed by the Institutional Animal Care and Use Committee. Tumor volume was no different among the Ang-1-transfected, vector-only-transfected, and parental-cell groups. Growth rate based on clonal variation was assessed using the MTT assay. Growth rates of the Ang-1 and -2 and empty-vector transfectants were not different (data not shown).

**Effect of Transfection on Ang Expression and Tumor Vessel Counts.** Immunohistochemical staining of harvested tumors confirmed high expression of Ang-1 in the group injected with the Ang-1-transfected cells and high expression of Ang-2 in the group injected with the Ang-2-transfected cells, relative to the expression of these compounds in the groups injected with the HT29 parental cell line or the pcDNA transfectants (data not shown). Immunohistochemical staining for CD31 revealed significantly more tumor vessels in the Ang-2-transfected group and significantly fewer tumor vessels

in the Ang-1-transfected group than in the other groups ( $P < 0.05$ ; Fig. 2A).

**Effect of Overexpression of Ang-1 and -2 on PCNA Expression and Endothelial Cell and Tumor Cell Apoptosis.** Immunohistochemical staining for PCNA (a measure of tumor cell proliferation) revealed that the number of PCNA-positive cells was greatest in the tumors from the Ang-2-transfected group, and least in tumors from the Ang-1-transfected group, relative to those in the other groups ( $P < 0.05$ ; Fig. 2B, Fig. 3). Immunofluorescent TUNEL staining of tumor sections from the four groups revealed slightly more TUNEL-positive cells in the tumors from the Ang-2 group than in the tumors from the other three groups (data not shown). However, in comparing these slides with the H&E slides, it became apparent that the large number of TUNEL-positive cells in the Ang-2 group was attributable to necrosis rather than apoptosis, because the tumors in the Ang-2 group were quite large and necrotic when they were harvested. Double staining (TUNEL and CD31) revealed no difference in EC apoptosis among the four groups (data not shown).

## Discussion

The importance of the Angs in embryonic angiogenesis has been clearly established (2, 6, 7). However, their role in tumor angiogenesis remains to be elucidated. Angs have been implicated in vessel cooption and survival of primary and metastatic tumors

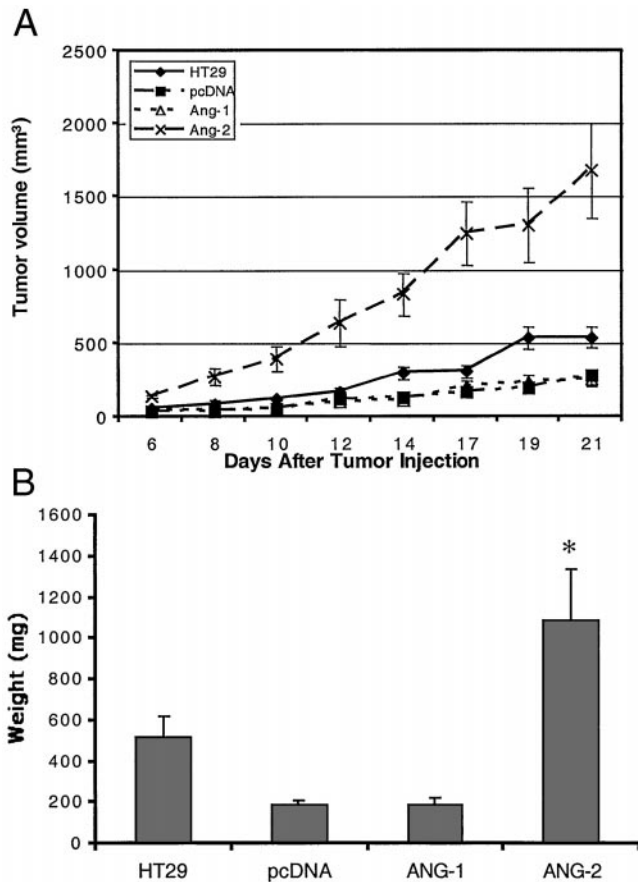


Fig. 1. Effect of Ang-2 overexpression on volume and weight of tumors formed by s.c. injection of HT29 human colon cancer cells in nude mice. Cells stably transfected with Ang-2 produced significantly larger (A) and heavier (B) tumors than did cells transfected with Ang-1 or pcDNA3.1 vector alone or parental HT29 cells (mean values  $\pm$  SE). Neither tumor volume nor tumor weight differed among the mice injected with Ang-1-transfected cells, with pcDNA mock-transfected cells, or with parental HT29 cells. \*,  $P < 0.05$  versus other groups (Student's *t* test).

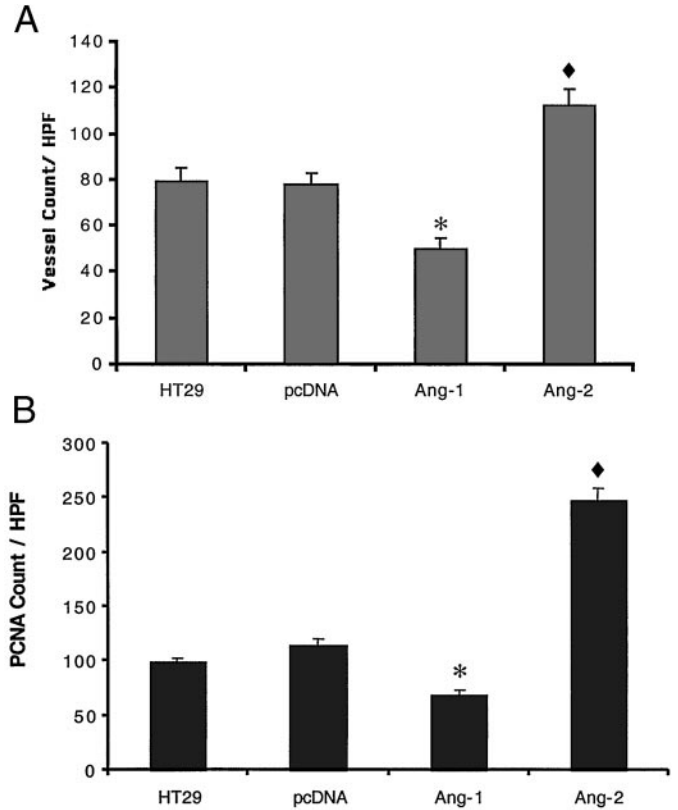


Fig. 2. Effect of Ang-1 and -2 transfection on tumor vessel count and proliferative index (PCNA) in tumors grown in nude mice. A, immunohistochemical staining for CD31 showed more vessels in the tumors from the Ang-2-transfected group than in the tumors from the other three groups (mean values  $\pm$  SE). Tumors produced by cells transfected with Ang-1 had significantly fewer blood vessels than the other groups. \*,  $P < 0.05$  versus HT29, pcDNA, and Ang-2 groups (Student's *t* test). ♦,  $P < 0.05$  versus HT29, pcDNA, and Ang-1 groups (Student's *t* test). B, immunohistochemical staining for PCNA showed higher tumor cell proliferation in the tumors from the Ang-2-transfected group than in the tumors from the other three groups (mean values  $\pm$  SE). Tumors produced by cells transfected with Ang-1 had less tumor cell proliferation than tumors from the other groups. \*,  $P < 0.05$  versus HT29, pcDNA, and Ang-2 groups. ♦,  $P < 0.05$  versus HT29, pcDNA, and Ang-1 groups.

(14). VEGF and Angs seem to play complementary and coordinated roles in vascular development to support new tumors. Briefly, low-level constitutive expression of Ang-1 by normal tissue stabilizes existing blood vessels. Ang-2 overexpression by newly formed tumor blood vessels leads to vessel destabilization and relative hypoxia. This hypoxia drives the release of VEGF, which leads to robust angiogenesis.

The current study was done to investigate the role of Angs in colon cancer by examining the effects of Ang-1 and -2 overexpression on tumor growth and angiogenesis in a xenograft model. We found that Ang-2 overexpression was associated with marked increases in tumor growth rate, vessel count, and proliferation. These observations are consistent with the proposed hypothesis that Ang-2, in the presence of VEGF, induces the formation of new blood vessels (1). Preliminary studies from our laboratory have shown that Ang-2 is constitutively expressed in colon cancer cells and in tumor endothelium.<sup>4</sup> VEGF is also expressed by all of the colon cancer cell lines studied to date (16, 17). In adults, Ang-2 is expressed primarily at sites of vascular remodeling, such as the ovaries, uterus, and placenta (2), where it is thought to block the constitutive stabilizing action of Ang-1. The destabilizing effects

<sup>4</sup> S. A. Ahmad and L. M. Ellis, unpublished observations.

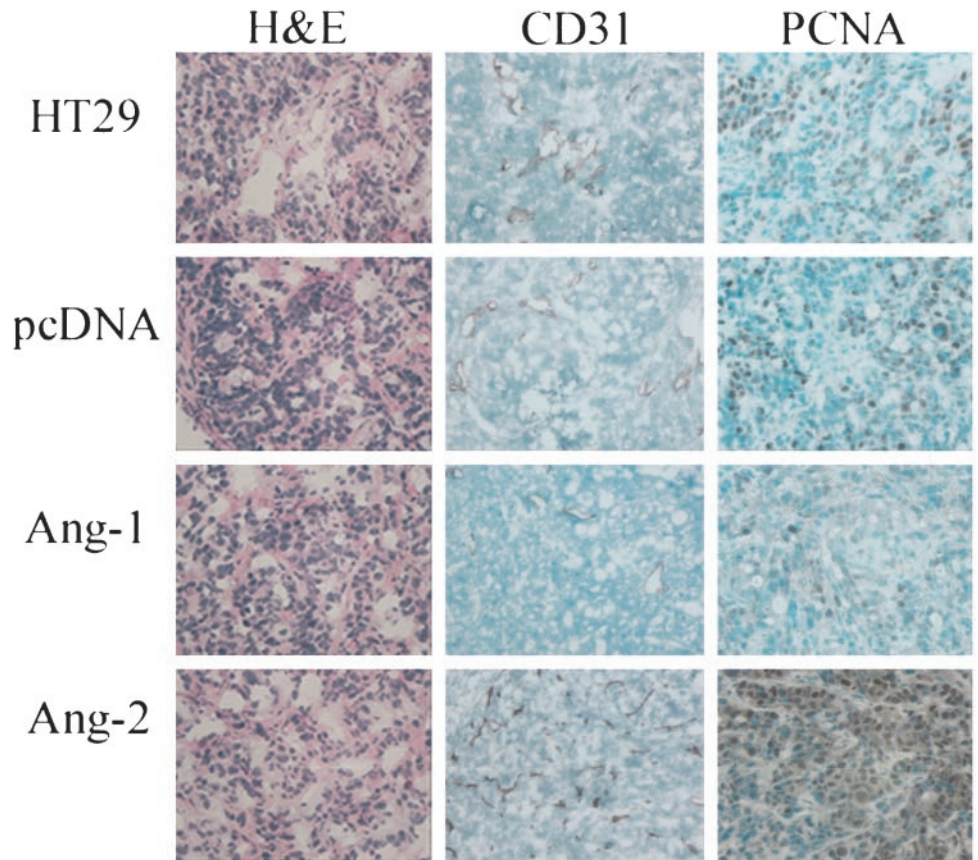


Fig. 3. Immunohistochemical analyses of tumors with increased Ang-1 and -2 expression. Representative photomicrographs demonstrate morphology (H&E;  $\times 100$ ), relative tumor vessel count (CD31;  $\times 100$ ), and tumor cell proliferation (PCNA;  $\times 100$ ) in tumors from mice injected with Ang-1 or -2 transfectants.

of Ang-2 in the absence of VEGF have been suggested to lead to vessel regression. In another study, Tanaka *et al.* (12) injected the livers of nude mice with human HuH7 hepatocellular cancer cells that overexpressed Ang-2; all of these mice died from extensive i.p. bleeding and the formation of large tumors. These findings support those from our current study that overexpression of Ang-2 leads to increased tumor growth.

We also demonstrated that overexpression of Ang-1 in colon cancer xenografts led to the production of fewer tumor vessels, a finding that is consistent with the known stabilizing action of Ang-1. Ang-1 acts via the Tie-2 receptor and is thought to help maintain and stabilize mature vessels by promoting interactions between ECs and surrounding support cells (2, 3, 18). Ang-1 also leads to increased Akt activation in ECs, thus enhancing survival signals (19). Ang-1 is widely expressed in adult tissues (12), a reflection of its role in maintaining previously developed and mature blood vessels. Thus, our finding that the tumors from the mice injected with Ang-1-transfected cells had fewer tumor vessels than the other groups probably reflects the stabilizing action of Ang-1 on the endothelium. We hypothesize that an overproduction of Ang-1 inhibits or slows angiogenesis because of this stabilizing effect. Conversely, overproduction of Ang-2, from any cell type, may induce angiogenesis and subsequent tumor growth in an *in vivo* system. This latter supposition was confirmed by our finding that the tumors consisting of Ang-2-transfected cells had not only more vessels but also higher tumor cell proliferation.

In summary, the overexpression of Ang-2 in human colon cancer cells led to increased angiogenesis and tumor growth in this murine model. A thorough understanding of the factors that initiate tumor angiogenesis may provide rational targets for therapy.

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